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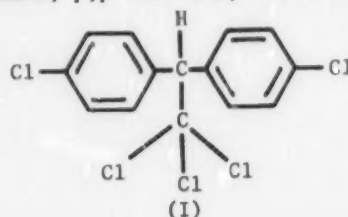
A STUDY OF INTERMOLECULAR COMPLEXES OF BIS (p-CHLOROPHENYL) ACETIC
ACID AND SOME BIOLOGICALLY SIGNIFICANT COMPOUNDS

Ralph T. Ross and Francis J. Biros

Environmental Protection Agency, Primate and Pesticides

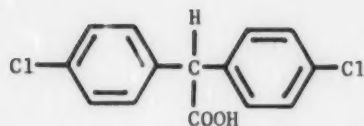
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The mode of action of chlorinated hydrocarbons has received much recent attention, and several elegant studies have attempted to relate binding and complexation phenomena with toxic effects.¹⁻⁷ The occurrence and persistence of the compound 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane, p,p'-DDT (I), in the environment, in addition



to the fact that its biochemical mode of action has not been unequivocally established, has resulted in a continued effort to determine its molecular interaction capabilities. To date, the study of complexation of p,p'-DDT with biological compounds has been limited due to its insolubility in aqueous systems. However, attempts have been made to establish, on a molecular level, the behavior of p,p'-DDT complexes in nonaqueous media, employing high resolution nuclear magnetic resonance (NMR) spectroscopy.^{5,8,9} The use of the NMR technique to study the behavior of such complexes permits more precise characterization of the possible molecular associations involved.

An important metabolite of p,p'-DDT is bis (p-chlorophenyl) acetic acid (p,p'-DDA), II. Since the specificity of biologically



(II)

active materials lies in the structure of the complexes they form with macromolecular receptors¹⁰, any qualitative data on the chemistry and function of agent-receptor complexes yields insight into the pharmacodynamic behavior of the agent molecule. Various chemical and physical techniques including spectrophotometry, microcalorimetry, chemical kinetics, structure-activity correlations involving chemical modifications of either the agent or the receptor, and electrophoresis have been used to establish specific molecular interactions of biochemical importance. A significant limitation of these methods is their inability to reflect relevant structural detail inasmuch as they measure large alterations in chemical and physical properties. Suitable models for this study are the complexes that p,p'-DDA may form with both bovine serum albumin (BSA) and human serum albumin (HSA). The vascular transport of p,p'-DDA may be an important step in the excretion of p,p'-DDT from the liver, the principal metabolic site.¹¹ The solubility of p,p'-DDA in slightly alkaline aqueous solutions and the ease of interpreting the NMR spectrum make this compound unique for the study of complexation with biological compounds. This study was made to directly test the existence of binding of p,p'-DDA with BSA and HSA and to examine the structure of the complexes.

EXPERIMENTAL

Proton NMR spectra were obtained on a Varian Associates HA-100 high resolution NMR spectrometer operating at 100 MHz. Chemical shifts were measured by a Hewlett Packard Model 5512A electronic frequency counter which displays the pen position in Hz from the lock signal, in this case an external capillary of tetramethylsilane. Temperature control and measurements were made with a Varian Associates V-4333 variable temperature probe and a V-4343 temperature controller.

All solutions for NMR studies were prepared with D₂O as solvent and p,p'-DDA concentrations are expressed in moles/liter (M). BSA and HSA concentrations are in percent w/v i.e., in grams per 100 ml final volume.

Except for the variable temperature studies, the NMR measurements were obtained at 31.5 ± 1.0°C. The spin-spin relaxation times,

T_2 , were obtained from a measurement of line width.¹² Each reported value of T_2 represents the mean of at least 6 measurements. All pD adjustments were made with $\text{DCl-D}_2\text{O}$ and $\text{NaOD-D}_2\text{O}$ solutions. pD measurements were made with a Beckman Expandomatic pH meter and are uncorrected for deuterium isotope effects.

Commercial sources used were as follows: D_2O , tetramethylsilane, and NaOD, Wilmad Chemical Co., Buena, N. J.; p,p'-DDA, Aldrich Chemical Co., Milwaukee, Wis.; crystallized bovine serum albumin and human serum albumin, Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

The NMR spectrum of 0.25 M solution of p,p'-DDA is shown in Figure 1. The aromatic protons are easily identified by their characteristic low field position at 7.54 ppm. The methine proton on the α carbon atom gives rise to a signal at 5.34 ppm. The remaining peak at 5.23 ppm is due to a water resonance arising from HDO generated by the replaceable hydrogen atoms of the p,p'-DDA, BSA, and HSA molecules.

To study the effects of BSA and HSA on the p,p'-DDA molecule, 0.25 and 0.40 M solutions of p,p'-DDA containing 0, 1.25, 2.50 and 4.00% of BSA and HSA respectively were examined. Figure 2 shows the results of a typical series of measurements. The relaxation rates of the aromatic protons are changed by a larger factor (x5) than the relaxation rate of the α -methine proton (x2). Thus the binding phenomenon may be interpreted in terms of preferential stabilization of the aromatic rings by binding sites on the BSA and HSA molecules.

Since the observed broadening may be nonspecific and may be due to alternate relaxation mechanisms such as intermolecular interactions between p,p'-DDA molecules, control experiments were performed on a series of solutions at varying concentrations of p,p'-DDA. Chemical shifts decreased as the concentration of p,p'-DDA increased, with a maximum shift of 18.2 Hz upfield for the aromatic resonances between 0.05 M and 2.00 M solutions. Differences in relaxation rates comparable to those observed on addition of BSA, however, were not observed.

The large upfield shift of the aromatic proton resonances may be attributable to extensive association of p,p'-DDA molecules at higher concentrations, resulting in an increase of shielding effects above and below the aromatic rings.¹³ Further, an apparent collapse of the AA'BB' multiplet¹⁴ of the two sets of aromatic protons to a single resonance line occurred at concentrations exceeding 0.20 M p,p'-DDA, under our conditions. Thus, specific molecular interactions involving solute or solvent molecules preferentially influence either the AA' or the BB' protons of the aromatic rings of

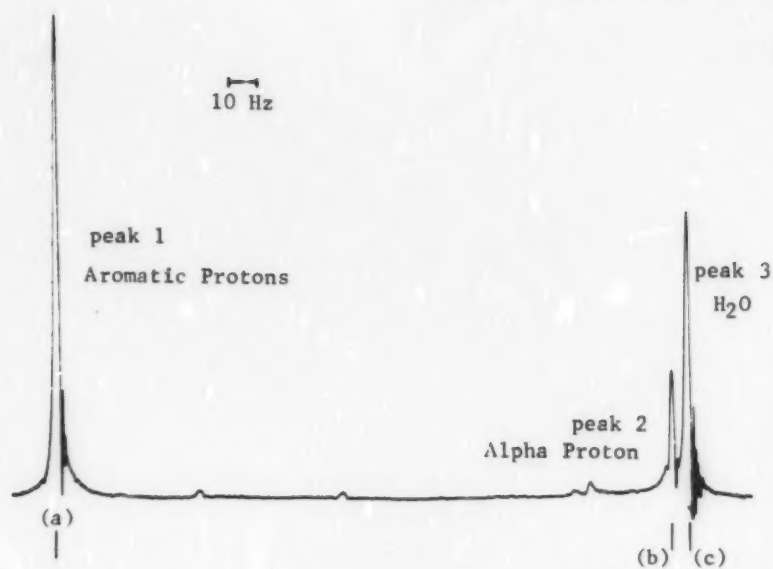


Figure 1

The NMR spectrum of 0.25 M solution of p,p'-DDA
 (a) Aromatic protons (753.7 Hz)
 (b) Benzylic proton (533.7 Hz)
 (c) Water protons (523.4 Hz)

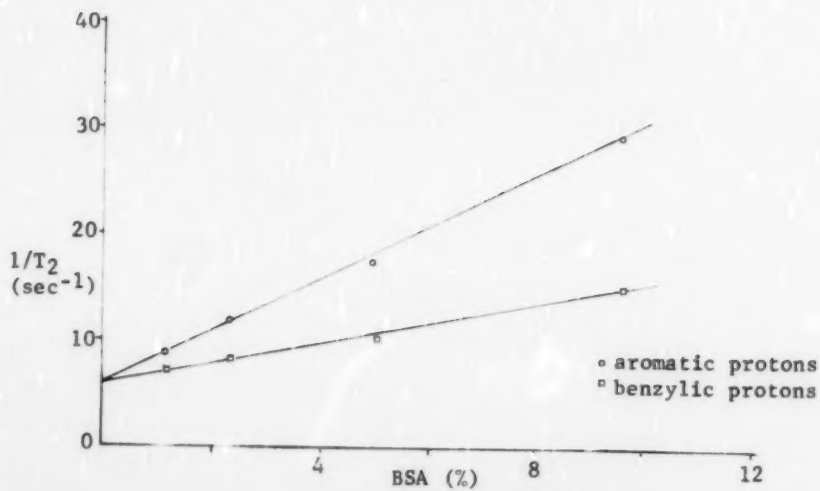


Figure 2

Plots of $1/T_2$ of a 0.25 M solution of p,p'-DDA vs BSA concentration.

Table 1. Effects of Ionic Strength on Chemical Shifts (Hz) and $1/T_2$ (Sec^{-1}) Values of p,p'-DDA Spectral Lines

NaCl Conc. M	Aromatic Protons			Tertiary Protons		
	Chemical Shift (Hz)	$1/T_2$ (Sec^{-1})		Chemical Shift (Hz)	$1/T_2$ (Sec^{-1})	
A. 0.25 M p,p'-DDA, 5% Albumin						
0.0	761.2	9.97	(0.18)	531.7	8.82	(0.53)
0.1	760.5	16.70	(0.42)	532.5	14.85	(0.16)
0.3	760.8	21.62	(0.22)	532.1	17.61	(0.09)
0.7	759.9	27.15	(0.27)	532.0	19.18	(0.23)
1.0	759.3	31.11	(0.5)	531.7	20.75	(0.31)
2.0	757.3	64.05	(0.31)	533.7	37.81	(0.11)
B. 0.25 M p,p'-DDA						
0.0	763.5	5.28	(0.62)	533.2	4.99	(0.10)
0.1	762.4	5.49	(0.35)	532.6	5.37	(0.35)
0.3	761.1	5.31	(0.49)	532.7	5.59	(0.26)
0.7	760.4	5.21	(0.3)	532.4	5.65	(0.08)
1.0	760.0	5.87	(0.22)	533.8	5.87	(0.32)
2.0	759.8	6.15	(0.48)	532.8	6.09	(0.46)

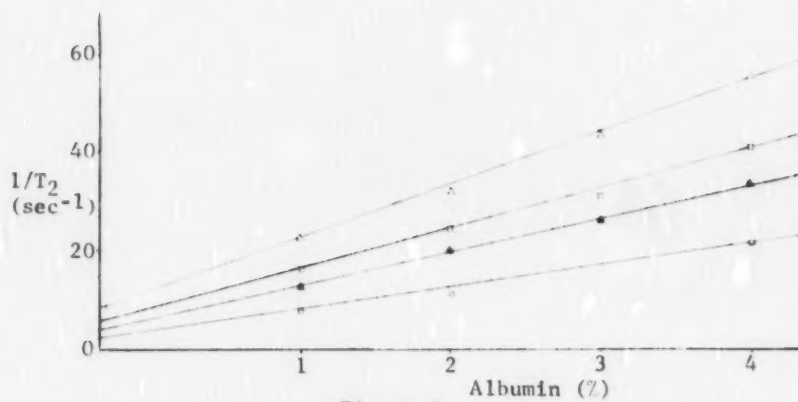


Figure 3

The effect of pD on the relaxation rates of 0.25 M solutions of p,p'-DDA at varying albumin concentrations (%). Δ p,p'-DDA·BSA pD 7.45; $\square$ p,p'-DDA·BSA pD 8.25; $\circ$ p,p'-DDA·BSA pD 9.70; $\blacktriangle$ p,p'-DDA·HSA pD 7.70.

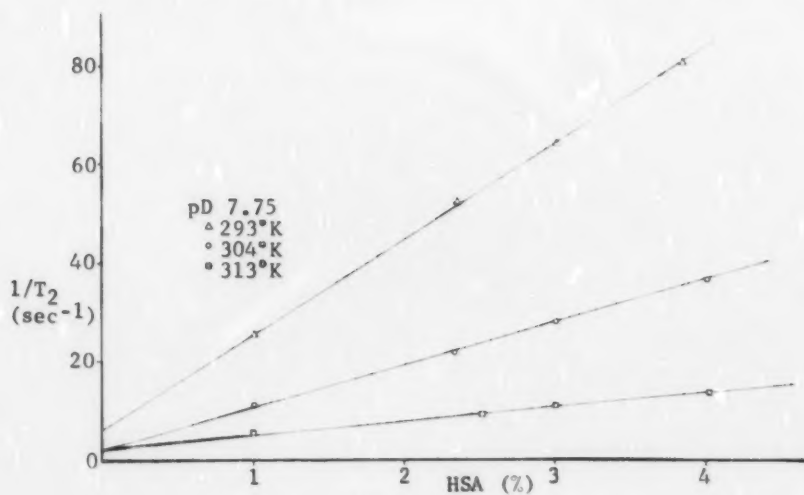


Figure 4

Temperature dependence of relaxation time vs HSA concentration.

p,p'-DDA at higher concentrations.^{15,16} This effect facilitated relaxation measurements of the aromatic ring protons.

Control experiments in which the chemical shifts of the AA' protons and the BB' protons were measured as a function of increasing BSA concentration have shown that virtually no contribution to the broadening of the aromatic proton resonances can be attributed to small internal chemical shift variations and incipient resolution of the complex, collapsed AA'BB' multiplet.

To study the effects of pD on the p,p'-DDA interactions with BSA and HSA, solutions of 0.25 M p,p'-DDA containing 0, 1.0, 2.5, 3.0, and 5.0% BSA and HSA were examined over the pD range 7.45-9.75. The measured relaxation rates were quite dependent on pD, as expected from a specific binding mechanism (Figure 3); the largest values are at pD 7.45 and decrease as the pD is increased, a finding consistent with the known behavior of albumin binding phenomena. BSA is known not to bind above pD 10.5 because of protein denaturation.¹⁷

The effect of ionic strength on the p,p'-DDA albumin interaction was studied with a solution of 0.25 M p,p'-DDA plus 5% BSA, together with a control solution of 0.25 M p,p'-DDA, in the presence of varying amounts of 0.0-2.0 M NaCl. The results of the line width measurements are given in Table 1. The increase in line width with increasing ionic strength is pronounced for p,p'-DDA·BSA but small for p,p'-DDA alone; the chemical shifts of the peaks of both solutions change in the same direction, upfield with increasing NaCl concentration.

These line width measurements are useful in deciding on a mechanism to explain p,p'-DDA·BSA binding. Since the energy of electrostatic attractions varies inversely with dielectric constant,¹⁸ one would expect an ionic bond between p,p'-DDA and BSA to be weakened by addition of salt. The reverse of this situation is observed. These findings suggest that p,p'-DDA·BSA interaction is hydrophobic, as expected, since the aromatic rings are the primary site of binding as discussed above.

The temperature dependence of the NMR spectra of p,p'-DDA with HSA is illustrated in Figure 4. Line widths for both p,p'-DDA·BSA and p,p'-DDA·HSA complexes were found to increase as the temperature decreased. This effect is probably due to an increase in the fraction of p,p'-DDA bound as the temperature decreases, since changes in line widths for unbound p,p'-DDA are not sufficient to account for the marked increase in relaxation rates.

To test the specificity of the binding sites on the albumin molecules, a series of inhibition studies was made. In these experiments an attempt was made to displace the p,p'-DDA from the

Table 2. Inhibition Studies with p-Chlorophenylacetic Acid; $1/T_2$ Values (Sec^{-1})

Sample Description	p,p'-DDA Spectral Lines		p-CPA Spectral Lines	
	Aromatic Protons	-CH-	Aromatic Protons	-CH-
0.25 M p,p'-DDA	3.11 (0.16)	2.97 (0.31)	-----	-----
1.20 M p-CPA	-----	-----	3.45 (0.19)	3.19 (0.25)
0.25 M p,p'-DDA	OVERLAP	3.79 (0.42)	OVERLAP	3.57 (0.11)
1.20 M p-CPA				
0.25 M p,p'-DDA 5% BSA	-----	-----	10.24 (0.48)	5.33 (0.35)
0.25 M p,p'-DDA 1.20 M p-CPA 5% BSA	8.86 ^a (0.45)	4.97 (0.22)	9.18 ^a (0.58)	4.45 (0.18)

^aSlight overlapping of peaks occurred; these values are estimates.

albumin by adding a large excess of another chemically analogous compound, p-chlorophenyl acetic acid (p-CPA).

In the NMR spectrum of p-CPA the methylene resonances are displaced upfield to 390.5 Hz relative to the tertiary proton in p,p'-DDA. Thus the relaxation rates of both these peaks in a mixture of these two compounds can be measured. Unfortunately, the aromatic proton resonances overlap somewhat and measurements of the relaxation rates of these peaks, where given, represent estimates. Table 2 gives the line widths of the resonance peaks of interest. The control experiments indicate that both p,p'-DDA and p-CPA bind to BSA. Furthermore, the spectrum of the p,p'-DDA, p-CPA and BSA mixture shows slightly less broadening of the p-CPA peaks and much less broadening of the p,p'-DDA peaks compared with controls. This difference may be due to a competition between p-CPA and p,p'-DDA for the binding sites on the serum albumin molecule.

SUMMARY

The results of this investigation have shown that with the use of high resolution NMR relaxation measurement studies, some insight into the importance of molecular binding of p,p'-DDA with BSA and HSA may be obtained. The binding phenomena may be interpreted in terms of preferential stabilization of the aromatic rings by binding sites on the BSA and HSA molecules. This was demonstrated by the greater relaxation rates found for the aromatic protons of the p,p'-DDA than those of the α -methine proton. These relaxation rates were quite pH dependent as would be expected from a specific binding mechanism. The binding of p,p'-DDA·BSA and p,p'-DDA·HSA is hydrophobic in nature since a direct relationship was found with ionic strength and relaxation times. The relaxation rates of both complexes increase with decrease in temperature.

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